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Vascular and Cardiac Impairments in Rats Inhaling Ozone and Diesel Exhaust Particles

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Table of Content

Methods

Ozone Generation

In 16-wk study, the ozone generator was a Gas Phase Titration Diluter built for the Environmental Protection Agency by Research Triangle Institute (Research Triangle Park, NC). Ozone was produced by passing clean dry air through a quartz tube, shielded by an adjustable aluminum housing, through a 9 inch Pen Ray mercury lamp which produces highly energetic atomic emission near 185 nm in addition to the resonant emission at 254-nm. The wavelengths near 185-nm are absorbed by molecular oxygen to produce oxygen atoms. Those oxygen atoms rapidly attach to oxygen via a termolecular reaction to form ozone. The ozone concentration was controlled by the adjustable housing which exposes more or less of the mercury lamp to the air stream, and by adjusting the air flow and dilution flow through the ozone generator. NOx concentrations were below 0.1 parts per billion (ppb) under the test conditions using air from the medical air compressor and about 1.0 ppm ozone (the ambient NOx during morning hours is about 2.5 ppb). The ozone was monitored continuously using a Photometric ozone analyzer (Model 400, API Teledyne, San Diego, CA). For acute exposure study ozone was generated from oxygen using a silent arc discharge ozone generator (model 3V1, Or Research Equipment Co., Phoenix, AZ). The concentrations of ozone were monitored continuously by ozone monitors (model 8002, Combustion Engineering Inc., Lewisburg, WV). The monitors were calibrated against a Dasibi transfer standard that is referenced to a primary ultraviolet ozone standard. The rats were exposed in 0.3 m^3 stainless steel chambers, one chamber for each concentration. To be consistent with the nose-only DEP and 16-wk ozone exposures, the rats were placed in nose-only tubes and onto stainless steel racks inside the exposure chambers. The mean ozone concentrations for the 0.5 ppm and 1.0 ppm chambers were 0.51 ppm and 1.00 ppm, respectively.

DEP Aerosol Generation

A string aerosol generator (Ledbetter et al., 1998) was used to suspend dry DEP to a 24-port nose-only inhalation chamber (EPA designed). The generator works by pulling string through a particulate filled reservoir where the particulate adheres to the string. The particulate laden string is then pulled into a discharge cavity where compressed air blows the particulate off the string into a horizontal mixing chamber, through a 2.5 micron cut point cyclone, through a vertical mixing/distribution chamber and finally into the inhalation chamber. String (South Maid 100% Mercerised Cotton Size 10; Coates & Clark; Greenville, NC) for the generator is stored on a fishing reel (Penn Model 109M; Penn Fishing Tackle Mfg.; Philadelphia, PA) inside a dry-air bathed container. The string passes through a Tygon™ tube that serves as the particulate reservoir, through a particulate discharge chamber and onto the take-up reel which pulls the string through the system. The take-up reel is controlled by a stepper motor that pulls the string in very small steps resulting in a slow but almost continuous advancement of the string through the system. The exposure concentration is controlled by adjusting the speed of the stepper motor.

As the string passes through the dust reservoir, two opposing squeezers slightly compress the reservoir walls to ensure maximum contact between particles and string and to prevent particle channeling inside the reservoir. Inside the discharge chamber an air jet blows the particulate off string into a horizontal cylindrical mixing chamber and through a cyclone (model URG-2000-30-EN; University Research Glassware; Chapel Hill, NC) which removes particles greater than 2.5 microns. The respirable particulate laden air enters a vertical cylindrical mixing/distribution chamber and then into the inlet of the inhalation chamber. To efficiently remove particulate from the string, the air supplying the generator must be as dry as possible. In addition the generator air supply supplies all the breathing air to the rats and therefore the air must also be of breathing quality. The air serving the generator is supplied by

a medical grade, oil-less compressor. A dryer /filtration system (Del-Monox; Deltech; Ocala, FL) removes almost all water from the air stream and also purifies the air of particles, organics and CO_2. Just prior to the air jet, the air is ionized using a 20 mCi Po-210 ionizer (Model P-2031-2000, NRD, LLC, Grand Island, NY). The ionizer generates both positive and negative ions into the air causing particles of opposite charge to attach to an ion thus removing the particles' charge. The estimated final humidity of incoming air to the system should be <5%.

The actual DEP concentration was determined by gravimetric samples taken approximately once per hour using 47 mm Teflon filters with a 1 micron pore size. Real time aerosol concentrations were determined using a Dustrack 8250 (TSI Inc. St. Paul, MN). Particle size of the DEP was determined using a Mercer 8-stage cascade impactor (In-Tox Products, Moriarty, NM).

16-Wk Episodic and Acute Exposure of Rats

The degree of pulmonary effect caused by DEP or ozone is likely dependent on the concentration of each in the present study. We presumed that the lung injury and inflammation resulting from ozone at ~0.5 ppm and DEP at ~2.0 mg/m^3 would be of similar magnitude over acute or episodic 16-wk exposure based on our own studies and the literature. Also, these concentrations might be achieved episodically over extreme environmental or occupational conditions. Therefore these target concentrations were chosen for the study. Each exposed animal was restrained in a conical, nose-only exposure plastic restrainer (Lab Products, Seaford, DE). All rats were acclimatized for 1 h to the nose-only inhalation tubes, 1 day prior to the start of exposure. In the 16-wk study, rats (n=20/group) were exposed for 5h/d 1d/wk to either ozone or DEP or to a combination of ozone+DEP for 16 wks. The desired chamber concentration of ozone was 0.5 ppm and DEP 2.0 mg/m^3. The mean temperature was 74^o F with a mean relative humidity of 35%. The chamber flows were approximately 12 l/m. In order to determine if changes seen following 16 wks episodic exposure were not due to an acute effect of the last

exposure, an additional study was performed where rats were exposed to air, ozone (0.5 or 1.0 ppm), or DEP (2.0 mg/m^3), 5h/d for two consecutive days and the lung injury, and lung as well as aorta biomarkers were analyzed one day post. Since, in general, in 16-wk study ozone+DEP exposure caused less severe changes when compared to individual exposures, this group was not examined in the acute study. Moreover, it was anticipated that two consecutive days of ozone exposure at 0.5 ppm might not result in detectable changes in lung as well as aorta biomarkers, and therefore, one group of rats was exposed to higher (1.0 ppm) concentration of ozone in place of ozone+DEP to serve as a positive control for lung effects for which detectable changes can be expected.

The exposure regimens for 16-wk study included, air only, ozone only, DEP only and ozone plus DEP (ozone+DEP). For ozone+DEP exposure, the ozone was introduced into the chamber air stream after the particle generator. This was done since the high back pressure of the aerosol generator caused the ozone generator to deliver inconsistent output. For acute study rats were exposed to air, ozone at two concentrations and DEP. As mentioned above, particulate concentration inside the nose-only chamber was determined gravimetrically approximately once/hour using a 47mm Teflon filter. Particle size was also monitored from the sample port using an 8-stage cascade impactor (Intox Products, Albuquerque, NM).

To theoretically estimate particle deposition that might occur as a result of DEP inhalation, we used a program developed by CIIT (now Hamner Institute, Durham, NC) and RIVM, Netherlands (Multiple Path Particle Deposition Model v1.11, CIIT/RIVM) for calculating tracheobronchial and pulmonary particle deposition in rats as described in our previous study (Kodavanti et al., 2008). Assuming DEP particle density being low (nearly 0.2-0.5), and considering particle size 0f 1.2 MMAD with GSD being 2.67, the pulmonary deposition fraction for rat is ~8%, tracheobronchial ~5% and head (nose) ~20%. A large amount of particles can be filtered through the nose (~20%). Using 8% pulmonary deposition fraction, and considering 200 ml tidal volume for a 300 g rat, the theoretical deposition fraction for pulmonary region was

calculated as ~10 μg, and pulmonary plus tracheobronchial deposition quantity was ~15 μg. Considering total deposition fraction (~33% which includes head, tracheobroncheal, and pulmonary), the amount of particles retained in the entire respiratory tract will be three times the pulmonary fraction.

Pulmonary Ventilation Monitoring

Pulmonary Ventilation was monitored by placing one rat in each of four whole body plethysmographic chambers (model PLY3223, Buxco Electronics, Wilmington, NC). The pressure signals from the plethysmographs were amplified (MAX1320 preamplifiers and interface; Buxco Electronics), and integrated by data analysis software (Biosystem XA for Windows, Buxco Electronics). Breathing variables determined were:

Variable	**Symbol**
Breathing Frequency	F
Tidal Volume	TV
Minute Volume	MV
Inspiratory Time	TI
Expiratory Time	TE
Peak Inspiratory Time	PIF
Peak Expiratory Time	PEF
Relaxation Time	RT
PenH (Enhanced Pause)	PenH
Pause	PAU

Pulmonary Testing Protocol

The pressure response of each Buxco chamber was calibrated each day just prior to the start of testing. To remove any testing bias, at least one rat/group was placed in each of the four Buxco boxes and the rats were tested in a pre-determined sequence so that some rats from

each group were tested at the beginning, middle and end of each testing period. The testing protocol was to place four rats in the Buxco chambers and allow them to acclimate for 1 minute after which data were collected every 10 seconds and averaged each minute for a total of five minutes. A five minute average was then calculated and used as the value.

16-Wk Study- The rats (6/group) were tested in the morning on the day prior to each exposure, just prior each exposure and the day following each exposure. In addition, the rats were monitored in the afternoon immediately after each exposure.

Acute Study- The rats (6/group) were tested in the morning for 2 days before the exposures, just prior to each exposure and the day following the exposures.

Blood Chemistry and Cytology

Aliquots of blood collected in tubes containing EDTA as an anticoagulant were analyzed for complete blood counts by a Beckman-Coulter AcT blood analyzer (Beckman-Coulter Inc., Fullerton, California). Each blood sample containing citrate anticoagulant was centrifuged at 4500 rpm for 10 min at 4 ^{o}C. Plasma was analyzed for fibrinogen using a commercially available kit (DiaSorin, Stillwater, MN). Angiotensin converting enzyme (ACE) activity was measured using reagents and controls from Sigma Diagnostics, St Louis, MO. Serum samples were analyzed for total cholesterol, low density lipoprotein, high density lipoprotein, and triglycerides using commercially available kits from Thermo Electron Corporation (Victoria, Australia). These assays were modified and adapted for use on the KONLAB clinical chemistry analyzer (Thermo Clinical Labsystems, Espoo, Finland). In both 16-wk and acute studies, plasma adiponectin was measured using a Milliplex Map kit (Millipore, Billerica, MA), following the manufacturer's instructions. Briefly, serum samples were diluted 1:1000, and incubated with the kit beads. After washing, detection antibodies and Streptavidin-Phycoerythrin were added sequentially. Samples were analyzed on Luminex 100 (Luminex Corp., Austin, TX) using Luminex 100IS software.

RNA Isolation and Real-Time PCR

Total lung, heart and thoracic aorta RNA was isolated from tissue frozen at -80ºC, with a commercially available RNeasy mini kit (Qiagen, Valencia, CA) using silica gel membrane purification. Total RNA from ~20mg tissue was resuspended in 40µl RNAse free water. For isolation of heart and aorta tissue RNA, proteinase-K digestion was performed which was a component of fibrous tissue RNA isolation kit (Qiagen, Valencia, CA). RNAse inhibitor was added and RNA yield was determined spectrophotometrically on the NanoDrop 1000 (Thermo Scientific, Wilmington, DE). Each RNA sample was diluted to a uniform concentration of 20 ng/µl and stored until RT-PCR reaction was carried out.

One-step real-time reverse transcription polymerase chain reaction (RT-PCR) was carried out using the SuperScript III One-step RT-qPCR kit from Invitrogen. All reactions were run in duplicate using 100ng total RNA. 18S ribosomal RNA, β-actin and/or HPRT were run as endogenous controls for each sample separately. RT-PCR was conducted on an ABI Prism 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). RT-PCR conditions were as follows: 20 minutes at 53ºC for reverse transcription, 2 minutes at 95ºC for inactivation of reverse transcriptase, followed by 40 cycles of 15 seconds at 95ºC and 45 seconds at 60ºC. All primers were purchased from ABI as inventoried TaqMan Gene Expression Assays, each containing a 6-carboxy-fluorescein (FAM dye) label at the 5' end. Data were analyzed using ABI sequence detection software (SDS), version 2.2. For each plate, cycle threshold (cT) was set to an order of magnitude above background. For each individual sample, target gene cT was normalized to control cT to account for variability in starting RNA amount. Expression of each exposure group was quantified relative to increase over air control, for acute and 16-wk studies.

Aorta LOX-1 and RAGE Protein Analysis

In a 16-wk study segments of intact thoracic aorta were washed with ice-cold PBS and

frozen at -80^{o}C until analysis. The tissues were homogenized in cell lysis buffer containing protease inhibitors mixture (Invitrogen, Inc.), and homogenates were spun at 2000 rpm to remove cell debris. For LOX-1, aorta homogenates were first quantified for total protein using a Bradford reagent kit (Biorad, Hercules, CA). Briefly, 5 µl of each sample was loaded for SDS-PAGE electrophoresis under reducing conditions. Membranes were blocked overnight at 4ºC in 5% blotto, incubated in rabbit polyclonal anti-mouse LOX-1 (reacts with rat LOX-1, 1:3000 dilution, Abcam, Cambridge, MA) and rabbit polyclonal anti-rat β-actin (for aorta blots - 1:2000, Abcam) for 1 hr at RT, and then incubated in anti-rabbit –HRP secondary (1:2000, Abcam) for 1 hr at RT. Bands were visualized with chemiluminescence and densitometry performed utilizing Image J software (NIH). For RAGE Western blotting was done using anti-RAGE (c20) polyclonal goat antibody (Santa Cruz Biotechnology #sc-8229). Blots were developed with a secondary donkey antibody towards goat IgG with a HRP conjugate (AbCam #ab-7125). The blots were then incubated in “SuperSignal West Pico” chemiluminescent substrate. Blots were imaged using a cooled CCD camera in a dark light box.

Results

Ozone and DEP Exposures

Each group of animals was exposed for 5h/d 1d/w for 16 consecutive weeks for 16-wk study and 2 consecutive days for acute study. In chronic study, the mean gravimetric diesel concentration for diesel only and diesel + ozone was 2.14 ± 0.66 and 2.19 ± 1.10 mg/m^3, respectively (Table 1). The mass medial aerodynamic diameter (MMAD) was 1.20 microns with a geometric standard deviation (GSD) of 2.67. The mean ozone concentration for ozone only and ozone+DEP was 0.403 ± .051 and 0.382 ± 0.064 ppm, respectively. Fluctuations occurred in DEP concentration, especially in combined (ozone+DEP) exposure groups during first three weeks, however, the chamber concentrations of DEP eventually stabilized near target values. Ozone tended to remain lower than the target level during most exposure days in both ozone alone and ozone+DEP groups. The mean temperature was 74^o F with a mean Relative humidity of 35%. The chamber flows were approximately 12 l/m. In the acute study the mean diesel concentration was 1.89 mg/m^3. The mass median aerodynamic (MMAD) was 0.86 microns with a geometric standard deviation (GSD) of 2.42.

Body Weights

Body weights were measured prior to and the day after each exposure. During nose only exposures, animals lose some weight from being confined in the nose-only tubes. On the day following exposure, the average weight loss for the air group, diesel only group, ozone only group and ozone+DEP group was -3 g, -4g, -9g and -8 g, respectively indicating that the ozone exposed animals were slightly more affected than the non-ozone exposed animals (Figure 1). During the 16-wk study, the air and diesel only groups gained slightly more weight (77 g and 81 g respectively) than the ozone only and diesel/ozone groups which gained 67 g and 71 g, respectively. Rat body weights were analyzed using a mixed model for repeated measures

procedure (SAS Version 9.2, SAS Institute Inc., Cary, NC) to determine the short and long term effects on each exposure group and differences between exposure groups. There was no significant effect of time x exposure, although some significant time related and also ozone exposure related differences were noted ($P \leq 0.05$).

Changes in Breathing Parameters

In order to understand how vascular alterations might relate to pulmonary functional and biochemical alterations, and if lung functional adaptation occurs following once weekly exposures, we analyzed breathing parameters over 16-wks. PenH, an index of labored breathing was found to be elevated only in rats exposed to ozone alone or ozone+DEP (Figure 2) suggesting that ozone independently affected PenH. This effect on PenH immediately following each weekly exposure from wk 3-16 was of smaller magnitude than the effect observed after first week of exposure, suggesting that, at least ozone was producing an acute pulmonary response following each exposure with partial adaptation in succeeding weeks (Figure 2). The DEP exposure did not affect PenH response at any time. The understanding of the nature of the lung effect and functional adaptation is critical in relating the differences between ozone and DEP-induced changes in the aorta in the 16-week study. The differences in the response between ozone and DEP on breathing parameters over 16 wks and how this may relate to changes in the aorta however, are difficult to explain within the context of the present experimental design (Figure 2). The breathing parameters data for acute exposure study are not shown.

Pulmonary and Systemic Alterations Following 16-Wk Exposure

A number of pulmonary and systemic biomarkers were assessed to understand their potential contribution in cardiac and vascular toxicity following episodic 16-wk exposure. BALF total cells, macrophages and neutrophils were quantified for determination of lung inflammation.

No significant changes were noted in total cells or alveolar macrophages; however a small but significant increase in neutrophils was noted following DEP exposure (Figure 3). A trend of an increase was also noted in rats exposed to ozone, but was statistically insignificant. This effect of DEP was not evident in rats exposed to ozone+DEP (Figure 3). To assess lung injury BALF protein, albumin and lactate dehydrogenase activities were analyzed. These injury markers were not increased by any of the exposure regimen; on the contrary the BALF LDH activity in ozone-exposed rats was lower than controls. Histologically particle laden macrophages were apparent in DEP-exposed rats with minimal chronic inflammation or fibrosis. A small degree of terminal bronchial thickening and inflammation were observed with ozone (Figure 4). Particle laden macrophages were also seen in airway associated lymphoid aggregates (Figure 4). Rats exposed to ozone+DEP showed mixed changes with a small degree of terminal bronchiolar thickening and also the presence of particle laden macrophages (not shown).

A complete blood count was performed to determine hematological parameters, white blood cells (total cells, lymphocytes and neutrophils), and platelets; however only small exposure related changes were noted in rats exposed to either ozone, DEP or ozone+DEP (Table 2). An increase in blood hemoglobin was noted in rats exposed to ozone or DEP but not ozone+DEP. A small decrease in circulating lymphocytes was seen in rats exposed to all three conditions when calculated as % of total white blood cells; however, this trend did not reach significance when absolute number of lymphocytes was considered. Blood platelets decreased only in DEP-exposed rats. A panel of clinical biomarkers, related to metabolic disorder and prothrombotic-state were also determined in the plasma/serum (glucose, total cholesterol, high density lipoprotein, low density lipoprotein, triglycerides, adiponectin, plasma fibrinogen and angiotensin converting enzyme activity), however, only a few changes were noted in some of these measures following 16-wk episodic exposure to ozone or DEP individually or in combination (Table 2). A very small but significant decrease in plasma fibrinogen was noted in rats exposed to DEP but not ozone or ozone+DEP. There was a trend of an increase in

adiponectin in all exposed rats; however this was not significant in any exposure groups.

Pulmonary and Cardiac Gene Expression Following 16-wk Exposure

No significant changes were noted in cardiac biomarker gene expression with any of the exposure conditions (Table 3). However, small but significant increases were noted in few biomarkers in the lung. TNF-α mRNA was increased ($P<0.05$) with ozone or DEP but not ozone+DEP. Lung MIP-2 expression was slightly but significantly induced by DEP ($P<0.05$). A small induction was noted in TF mRNA in rats exposed to ozone+DEP while tPA increased in rats exposed to ozone or DEP alone and PAI-1 in DEP exposed rats (Table 3).

Acute Ozone and DEP-Induced Pulmonary Injury and Systemic Alterations

As expected, no significant changes were seen at 0.5 ppm ozone in lung inflammation and injury markers as assessed in BALF (Figure 5). No significant changes were noted in the DEP-exposed rats either. However all lung injury and inflammation markers in BALF (total cells, neutrophils, protein, albumin) but not lactate dehydrogenase activity, increased significantly in rats exposed to 1.0 ppm ozone (Figure 5). No other major changes were noted in systemic CBC biomarkers in acute study. Blood platelets tended to be higher in rats exposed to ozone and DEP but not significantly (Table 4). The levels of plasma adiponetin tended to decrease in rats exposed acutely to ozone and DEP, but this trend too was not statistically significant (Table 4).

Acute Ozone and DEP-Induced Changes in the Lung and Aorta mRNA Biomarkers

Most mRNA biomarkers examined in the subchronic study were also examined in the lung and the aorta in the acute study. Increases were noted in expression of HO-1 at 1.0 ppm ozone; the levels tended to be high in DEP-exposed rats too but did not reach significance (Figure 6). Ozone also caused significant increase in PAI-1 expression at 0.5 ppm (Figure 6).

DEP but not ozone increased expression of MIP-2, and t-PA in the lung. Surprisingly, DEP also increased LOX-1 and RAGE expression in the lung but not ozone.

In the aorta, significant increases were noted in the ET-1 expression with 1.0 ppm ozone and with DEP. ETR-B expression also increased with 1.0 ppm ozone in the aorta (Figure 7). Because the levels of aorta LOX-1 and HMGB-1 mRNA in 0.5 ppm ozone-exposed rats tended to be lower than air controls whereas that of DEP tended to be higher, the comparison between 0.5 ppm ozone and DEP yielded statistically significant difference. However, no consistent changes could be discernible in LOX-1, RAGE and HMGB-1 expression relative to air controls following acute exposure in the aorta (Figure 7).

Tables and Table Footnotes

Supplemental Material, Table 1

Daily mean ozone and DEP concentrations in each chamber during 5h/d, 1d/wk 16-wk exposure periods.

Exposure	Ozone alone	DEP alone	Ozone + DEP	
Week	ppm ozone	mg/m^3 DEP	ppm ozone	mg/m^3 DEP
1	0.46 ± 0.01	1.96 ± 0.29	0.46 ± 0.01	2.16 ± 0.61
2	0.40 ± 0.01	1.17 ± 0.42	0.37 ± 0.01	3.53 ± 0.81
3	0.39 ± 0.01	2.65 ± 1.40	0.38 ± 0.00	4.30 ± 3.96
4	0.38 ± 0.00	2.13 ± 0.65	0.38 ± 0.01	1.49 ± 0.57
5	0.38 ± 0.01	1.35 ± 0.31	0.36 ± 0.00	1.23 ± 0.59
6	0.38 ± 0.01	1.84 ± 0.49	0.35 ± 0.00	1.11 ± 0.51
7	0.37 ± 0.00	2.52 ± 0.35	0.34 ± 0.00	2.10 ± 0.94
8	0.36 ± 0.01	2.30 ± 1.00	0.34 ± 0.02	1.96 ± 0.61
9	0.37 ± 0.01	2.65 ± 0.74	0.34 ± 0.01	2.46 ± 0.57
10	0.38 ± 0.02	2.08 ± 0.33	0.35 ± 0.02	2.12 ± 1.15
11	0.34 ± 0.01	2.13 ± 0.34	0.31 ± 0.01	2.39 ± 0.08
12	0.36 ± 0.01	2.03 ± 0.88	0.32 ± 0.01	1.89 ± 0.63
13	0.37 ± 0.00	2.32 ± 0.12	0.34 ± 0.00	2.37 ± 0.56
14	0.38 ± 0.01	2.16 ± 0.25	0.35 ± 0.01	2.05 ± 0.44
15	0.40 ± 0.01	2.43 ± 0.36	0.36 ± 0.01	2.31 ± 0.43
16	0.46 ± 0.01	2.20 ± 0.58	0.44 ± 0.01	2.40 ± 0.61
17*	0.49 ± 0.01	2.32 ± 0.12	0.49 ± 0.01	2.27 ± 0.23

Supplemental Material, Table 1 Footnotes: *Seventeen exposures were conducted to accommodate necropsies for over two weeks time, while each group of animals was exposed for a total of 16 exposures only. Daily mean concentrations for DEP were calculated from hourly measurements during each exposure day. Daily mean ozone concentrations were calculated from continuous monitoring of ozone during each 5 hour exposure period.

Supplemental Material, Table 2

Alterations in hematological parameters and plasma biomarkers of cardiovascular disease as well as metabolic syndrome in rats following episodic exposure to ozone, DEP or ozone+DEP.

Tissue	Biomarker	Air	Ozone, 0.5 ppm	DEP, 2.0 mg/m^3	Ozone+DEP
Whole Blood	Red blood cells/ml x 10^9	7.28 ± 0.06	7.25 ± 0.06	7.33 ± 0.09	7.32 ± 0.08
	White blood cells/ml x 10^6	1.65 ± 0.12	1.55 ± 0.07	1.46 ± 0.29	1.60 ± 0.08
	Hematocrit, %	37.00 ± 0.33	37.05 ± 0.35	37.82 ± 0.52	37.56 ± 0.50
	Hemoglobin, g/dl	12.15 ± 0.13	13.38 ± 0.07*	13.38 ± 0.16*	12.62 ± 0.19
	Platelets/ml x 10^9	743 ± 6	737 ± 14	644 ± 11*	732 ± 18
	Lymphocytes (%)	88.05 ± 1.64	67.45 ± 1.89*	62.94 ± 0.76*	80.82 ± 1.67*
	Lymphocytes/ ml x 10^6	1.45 ± 0.11	1.05 ± 0.05	0.90 ± 0.18	1.30 ± 0.06
Citrated plasma/ serum	ACE Activity, U/L	92.6 ± 2.2	89.1 ± 2.0	92.7 ± 2.7	93.9 ± 1.9
	Fibronogen, mg/dl	204.5 ± 1.5	202.1 ± 3.0	199.4 ± 1.1*	204.5 ± 1.7
	Total cholesterol, mg/dl	97.2 ± 1.8	99.4 ± 1.8	100.2 ± 1.4	94.6 ± 1.6
	Glucose, mg/dl	194.7 ± 7.5	183.5 ± 3.4	200.2 ± 5.6	204.8 ± 15.8
	High density lipoprotein, mg/dl	27.5 ± 0.8	29.4 ± 0.8	28.8 ± 0.5	28.8 ± 0.8
	Low density lipoprotein, mg/dl	11.4 ± 0.4	11.0 ± 0.4	12.1 ± 0.3	10.2 ± 0.2
	Triglycerides, mg/dl	46.7 ± 3.3	50.1 ± 4.6	46.9 ± 4.0	49.6 ± 3.8
	Adiponectin, µg/ml	111.9 ± 35.7	175.4 ± 70.9	160.6 ± 44.9	174.5 ± 33.9

Supplemental Material, Table 2 Footnotes: Blood was collected through abdominal aorta directly into vaccutainer tubes containing citrate or sodium EDTA as anticoagulants, or into serum separator tubes with no anticoagulant. CBC were performed with blood samples collected in EDTA tubes. Plasma/serum samples were used for other biomarkers. Each value represents mean SE of n=12 replicates for CBC and n=6 replicates for markers analyzed in citrated plasma. Dunn's post-hoc test was used to determine the level of significance for fibrinogen while Holm-Sidak method was used for all other significant comparisons. *Indicate significant difference from air controls ($P \leq 0.05$).

Supplemental Material, Table 3

Histological evaluation of multiple tissues of rats exposed episodically to filtered air, ozone, DEP and ozone+DEP for 16 weeks.

Exposure	Lung			Mononuclear cell infiltration		
	Alveolar histiocytosis with particles	Alveolar duct epithelial hyperplasia	Pigmented cells in bronchus-associated lymph nodes	Heart	Aorta	Spleen
Air	0/20 (0.00)*	0/20 (0.00)	0/20 (0.00)	6/15 (0.40)	0/12	0/6
Ozone, 0.5 ppm	0/20 (0.00)	16/20 (0.80)	0/20 (0.00)	3/15 (0.20)	0/12	0/6
DEP, 2 mg/m^3	19/20 (0.95)	0/20 (0.00)	13/20 (0.65)	7/15 (0.47)	0/12	0/6
Ozone+ DEP	20/20 (1.0)	20/20 (1.00)	15/20 (0.75)	4/15 (0.27)	0/12	0/6

Supplemental Material, Table 3 Footnotes: *The values show number of animals affected/number of animals examined in 16-wk study. The values in parenthesis show mean severity score per animal based on scale of 0 being no abnormality to 4 being most severe. Because all affected animals depicted the severity score of 1, the values in parenthesis also reflect the faction of animals affected by exposure.

Supplemental Material, Table 4

Relative mRNA expressions in the lung and the left ventricular tissues following episodic exposure of rats to ozone, DEP or ozone+DEP for 16 weeks.

Tissue	mRNA	Air	Ozone, 0.5 ppm	DEP, 2.0 mg/m^3	Ozone+DEP
Lung	HO-1	1.09 ± 0.13	1.26 ± 0.17	1.52 ± 0.16	1.42 ± 0.25
	TNF-α	1.07 ± 0.07	1.75 ± 0.18*	1.67 ± 0.10*	1.42 ± 0.16
	MIP-2	1.05 ± 0.10	1.43 ± 0.14	1.57 ± 0.08*	1.23 ± 0.14
	TF	0.90 ± 0.08	0.96 ± 0.06	0.97 ± 0.05	1.37 ± 0.14*
	t-PA	1.04 ± 0.07	1.40 ± 0.09*	1.48 ± 0.09*	1.18 ± 0.07
	PAI-1	0.95 ± 0.07	1.36 ± 0.13	1.50 ± 0.23*	1.38 ± 0.17
	vWf	1.07 ± 0.11	1.33 ± 0.05	1.01 ± 0.07	0.92 ± 0.10
	Thbd	1.00 ± 0.07	1.28 ± 0.05	1.10 ± 0.09	0.98 ± 0.08
	ET-1	1.05 ± 0.11	1.06 ± 0.07	1.34 ± 0.12	1.46 ± 0.18
	ETR-A	1.02 ± 0.07	1.25 ± 0.04	1.17 ± 0.05	1.17 ± 0.09
	ETR-B	1.04 ± 0.07	1.33 ± 0.11	1.35 ± 0.08	1.36 ± 0.06
	ANP	1.33 ± 0.38	1.21 ± 0.44	2.88 ± 1.14	2.75 ± 0.91
	BNP	1.20 ± 0.21	1.17 ± 0.19	1.76 ± 0.65	2.41 ± 0.44
	RAGE	1.03 ± 0.06	1.29 ± 0.05	1.15 ± 0.05	1.19 ± 0.07
	HMGB-1	0.99 ± 0.09	1.13 ± 0.05	1.13 ± 0.08	1.04 ± 0.06
Heart	HO-1	0.98 ± 0.08	1.15 ± 0.11	1.27 ± 0.12	1.70 ± 0.31
	TNF-α	0.95 ± 0.08	1.16 ± 0.08	0.99 ± 0.06	0.96 ± 0.14
	MIP-2	1.14 ± 0.11	1.38 ± 0.11	1.54 ± 0.17	1.82 ± 0.25
	TF	1.02 ± 0.03	1.21 ± 0.13	1.14 ± 0.25	1.37 ± 0.17
	t-PA	1.01 ± 0.03	1.10 ± 0.08	1.07 ± 0.05	1.22 ± 0.26
	PAI-1	0.94 ± 0.08	0.78 ± 0.04	0.92 ± 0.09	0.85 ± 0.19
	vWf	0.99 ± 0.03	1.06 ± 0.08	1.06 ± 0.09	1.04 ± 0.19
	Thbd	1.04 ± 0.07	1.15 ± 0.06	1.18 ± 0.11	1.04 ± 0.10
	ET-1	1.01 ± 0.09	1.10 ± 0.05	1.31 ± 0.18	1.23 ± 0.15
	ETR-A	1.02 ± 0.05	0.90 ± 0.11	1.08 ± 0.07	0.99 ± 0.09
	ETR-B	0.96 ± 0.05	1.08 ± 0.06	1.34 ± 0.12	1.46 ± 0.18
	ANP	1.16 ± 0.20	1.00 ± 0.13	1.79 ± 0.33	2.73 ± 1.20
	BNP	0.99 ± 0.04	0.90 ± 0.06	0.96 ± 0.11	1.17 ± 0.26
	RAGE	0.99 ± 0.04	0.95 ± 0.02	0.83 ± 0.05	1.04 ± 0.15
	HMGB-1	1.00 ± 0.04	0.95 ± 0.07	1.60 ± 0.33	1.19 ± 0.08

Supplemental Material, Table 4 Footnotes: Relative fold change for each animal was computed by normalizing to average value of air controls. HO-1=hemeoxygenase-1; TNF-α=tumor necrosis factor-α; MIP-2=macrophage inflammatory protein-2 (a human analog of

interleukin-8); TF=tissue factor; t-PA=tissue plasminogen activator; PAI-1=plasminogen activator inhibitor; vWf=von Willebrand factor; Thbd=thrombomodulin; ET-1=endothelin-1; ETR-A=endothelin receptor-A; ETR-B=endothelin receptor-B; ANP=atrial natriuretic peptide; BNP=brain natriuretic peptide; RAGE=receptor for advanced glycation endproduct; HMGB-1=high mobility group box-1. All rats were exposed nose-only, 5 hours/day, one day/wk for 16 wks. Values represent mean ± SE of 5-6 rat tissues, each analyzed in duplicate. *Denotes statistically significant difference from air controls ($P \leq 0.05$). The statistical analysis for TNF-α, MIP-2 and tPA was done by one-way ANOVA followed by post hoc comparison using Holm-Sidak method. For TF and PAI-1 Kruskal-Wallis one-way ANOVA was performed on ranks followed by post hoc comparison using Tukey's method.

Supplemental Material, Table 5

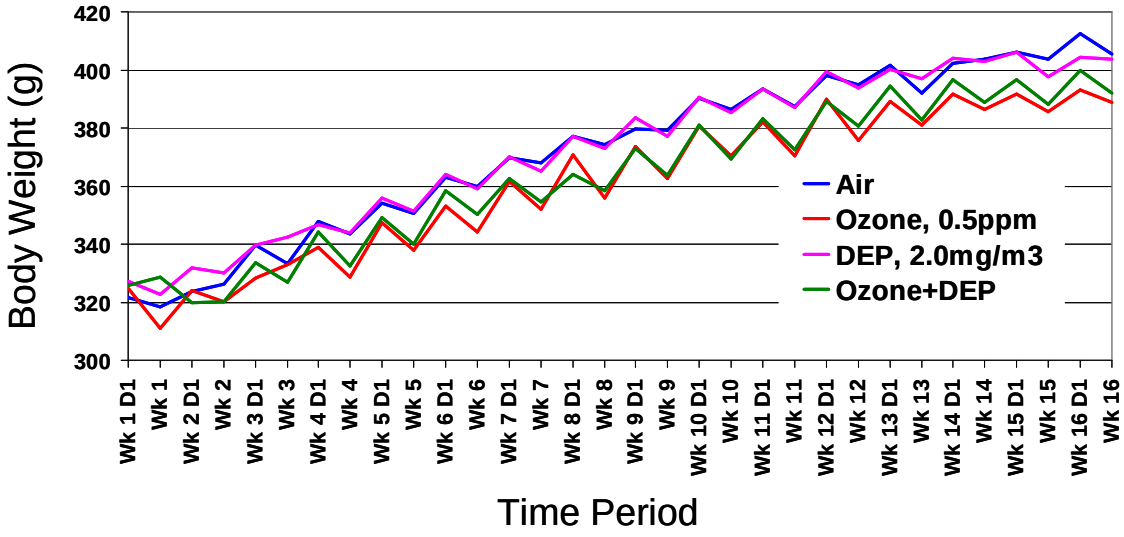
Hematological parameters, and plasma adiponectin in rats exposed acutely (5h/d x 2 days) to ozone or DEP.

Tissue	Biomarker	Air	Ozone, 0.5 ppm	Ozone, 1.0 ppm	DEP, 2.0 mg/m^3
Whole Blood	Red blood cells/ml x 10^9	6.69 ± 0.28	7.45 ± 0.11	7.39 ± 0.22	7.34 ± 0.11
	White blood cells/ml x 10^6	1.95 ± 0.69	1.84 ± 0.75	2.22 ± 0.44	2.03 ± 0.31
	Hematocrit, %	37.3 ± 0.8	39.8 ± 0.6	39.5 ± 1.3	39.3 ± 0.6
	Hemoglobin, g/dl	13.5 ± 0.5	14.5 ± 0.2	15.0 ± 0.3	14.3 ± 0.1
	Platelets/ml x 10^9	706 ± 95	869 ± 48	804 ± 108	830 ± 10
	Lymphocytes (%)	72.68 ± 2.39	65.40 ± 5.53	68.12 ± 4.02	77.50 ± 2.53
	Lymphocytes/ ml x 10^6	1.43 ± 0.51	1.34 ± 0.68	1.50 ± 0.28	1.58 ± 0.26
Citrated plasma/ serum	Adiponectin, μg/ml	201.8 ± 42.6	144.6 ± 33.8	122.2 ± 48.6	161.3 ± 59.1

Supplemental Material, Table 5 Footnotes: Blood was collected through abdominal aorta directly into vaccutainer tubes containing citrate or sodium EDTA as anticoagulants. CBC was performed using blood collected in EDTA tubes. Plasma samples were prepared by centrifugation of blood in citrated tubes. Each value represents mean SE of n=6/group. No exposure related changes were significant at $P \leq 0.05$ in these biomarkers.

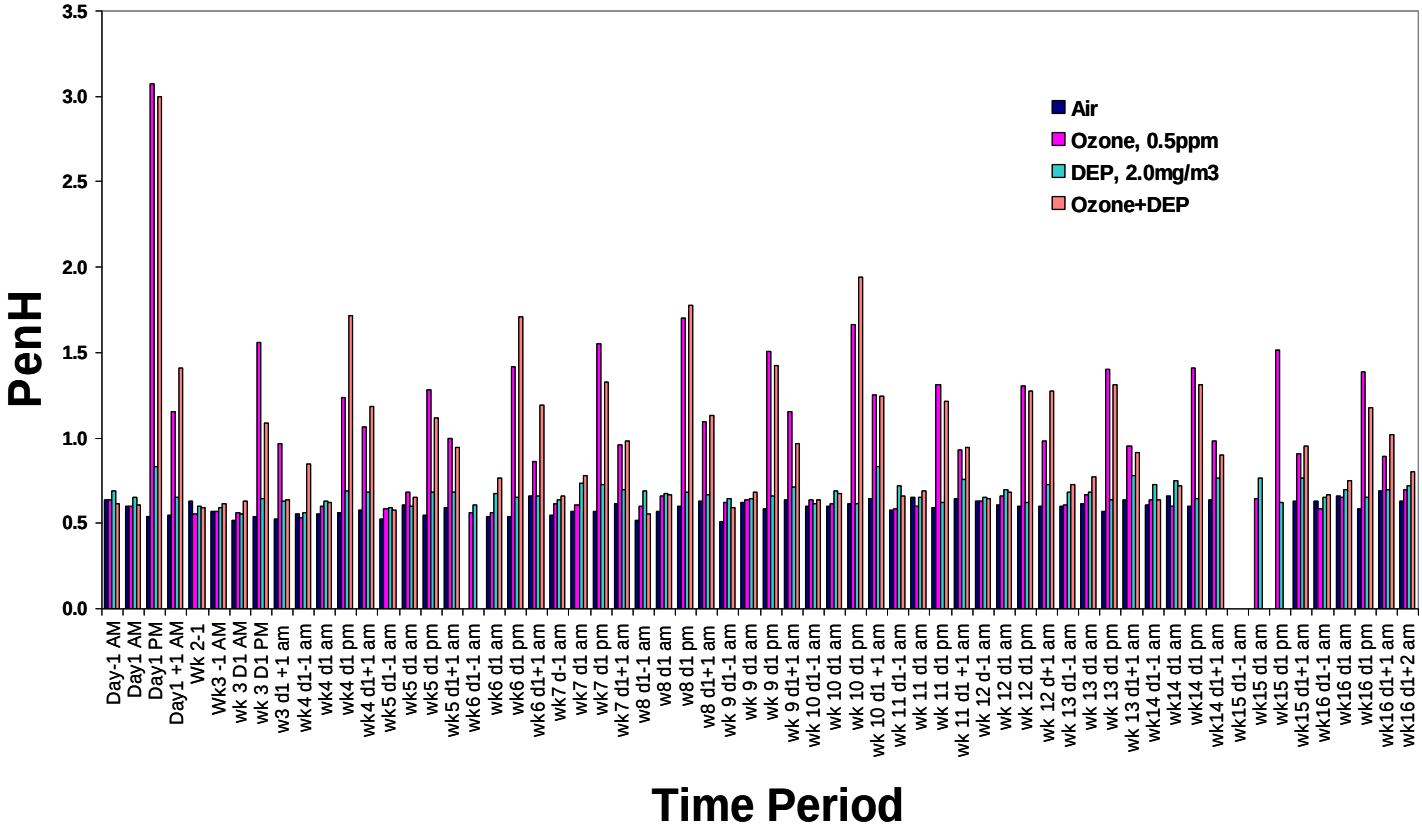
Figures and Figure Legends

Supplemental Material, Figure 1



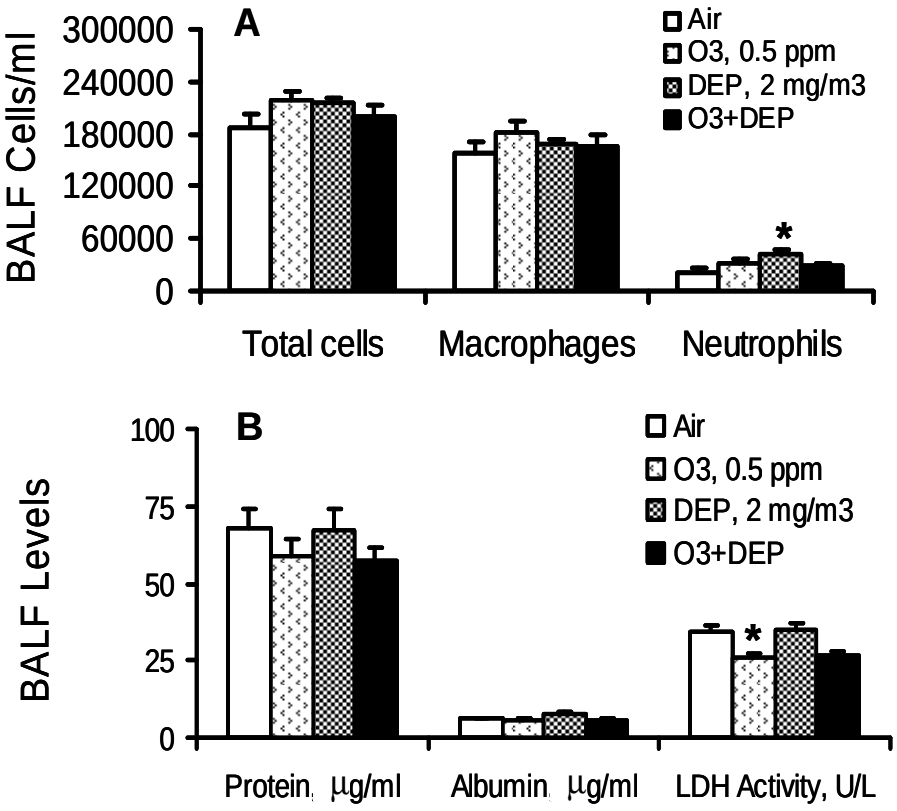
Supplemental Material, Figure 1 Legend: Body weights prior to and one day following each weekly exposure of rats to ozone, DEP, or ozone+DEP. Rats were weighed prior to and one day following each weekly exposure. Note that Wk 1 D1…. on x-axis represents values taken each week prior to the start of exposure whereas, Wk 1… represents body weights determined at the same time next day morning. Each bar represents the mean ± SEM for 12 animals.

Supplemental Material, Figure 2



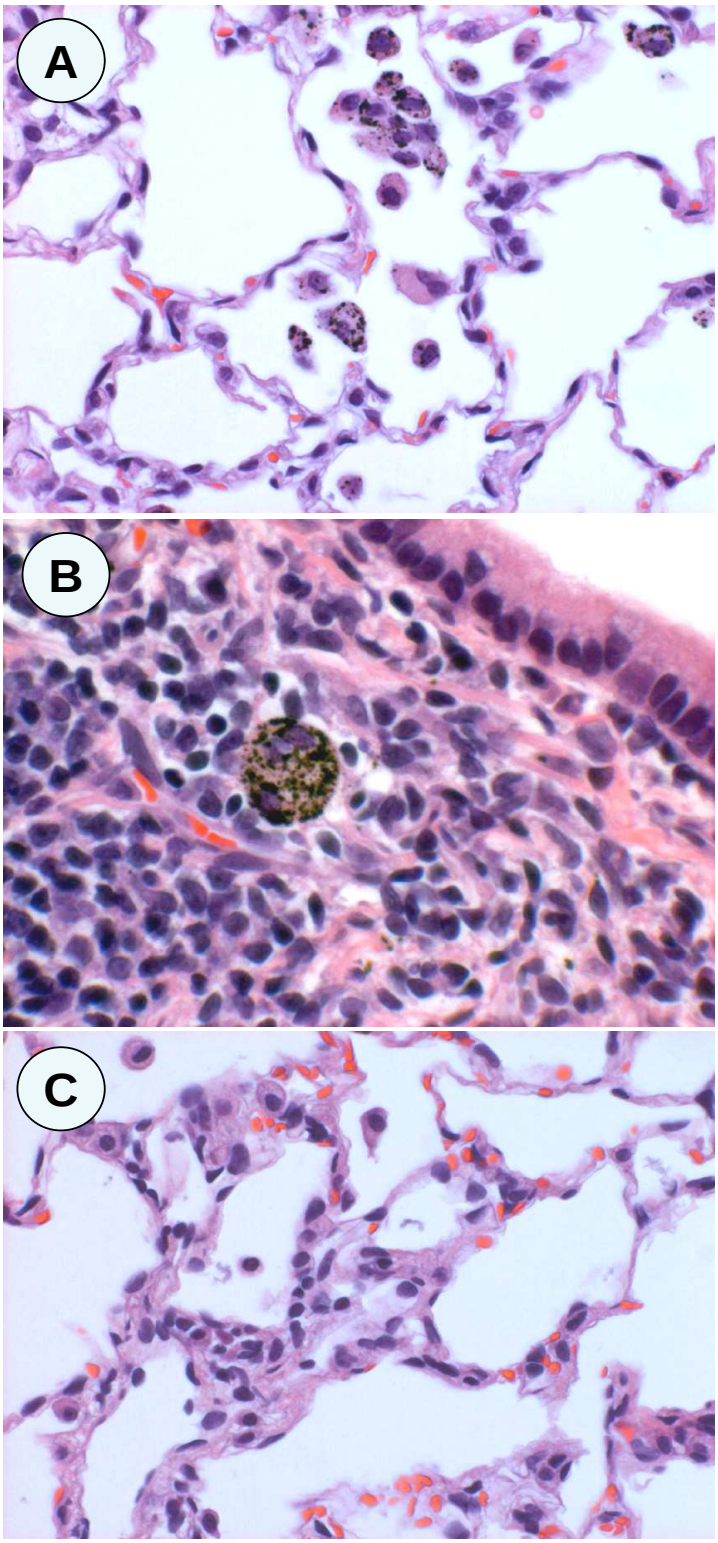
Supplemental Material, Figure 2 Legend: PenH, an index of labored breathing examined in rats one day prior to, the morning of exposure, immediately after and one day following each weekly exposure to ozone, DEP, or ozone+DEP. Note that Day-1AM, Day1 AM, Day1 PM and Day1+1 AM on X-axis represents measurements one day prior to exposure (morning), the day of exposure (morning prior to start of exposure), after exposure in the afternoon (the day of exposure) and one day following exposure (morning) for week 1. Each weekly exposure on x-axis is shown by the number of week. Each bar represents the mean ± SEM for 6 animals.

Supplemental Material, Figure 3



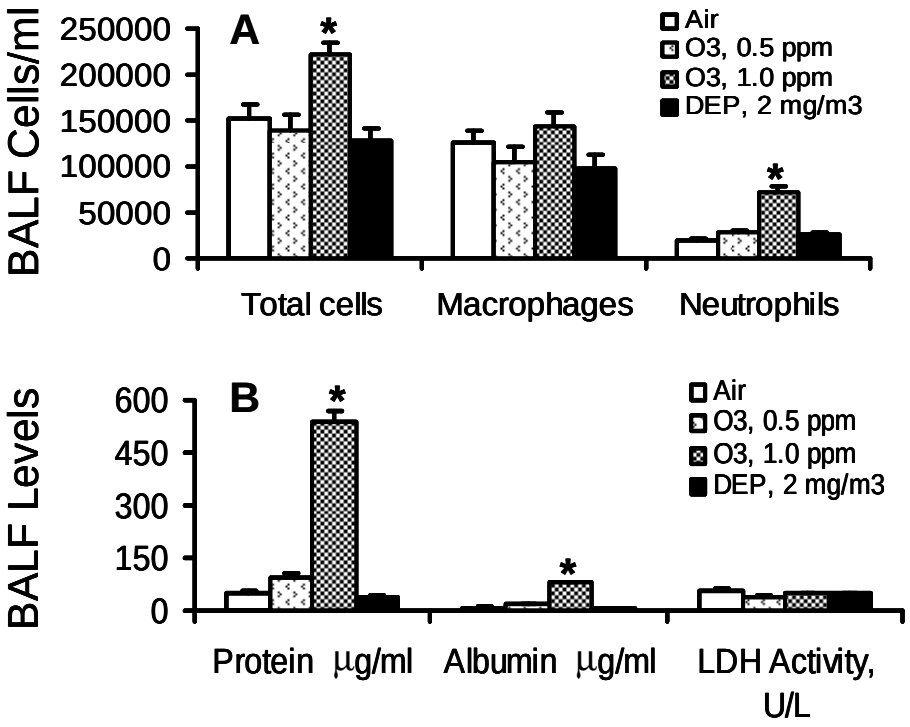
Supplemental Material, Figure 3 Legend. Pulmonary inflammation and injury parameters were determined in BALF following episodic 16-wk exposure to ozone, DEP or ozone+DEP in rats. BALF was assessed for inflammatory cells (A) and injury markers such as protein, albumin, and LDH activity (B). Each bar represents the mean ± SEM for 6 animals. One-way ANOVA followed by Holm-Sidak method was selected for neutrophil data while Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's post-hoc test was employed for LDH activity. *Indicate significant exposure effect relative to air control ($P \leq 0.05$).

Supplemental Material, Figure 4



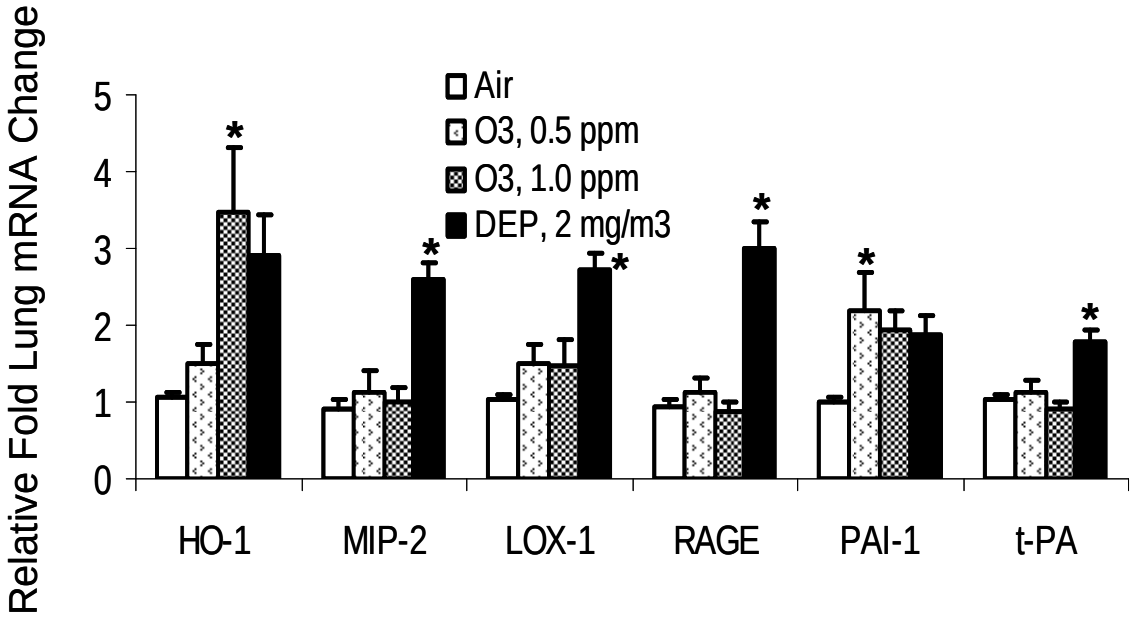
Supplemental Material, Figure 4 Legend: Histological alterations in the lung following exposure to ozone or DEP in rats. Particle laden macrophages were apparent without other abnormalities following DEP exposure (A). DEP-loaded macrophages were also apparent in airway associated lymphoid aggregates (B). The effect of ozone was mild and focal. Terminal bronchiolar thickening and mild inflammation were observed following ozone exposure (C). Exposure to ozone+DEP caused mild terminal bronchiolar thickening. Particle-laden macrophages were also apparent but the lesion severity was not exacerbated (not shown).

Supplemental Material, Figure 5



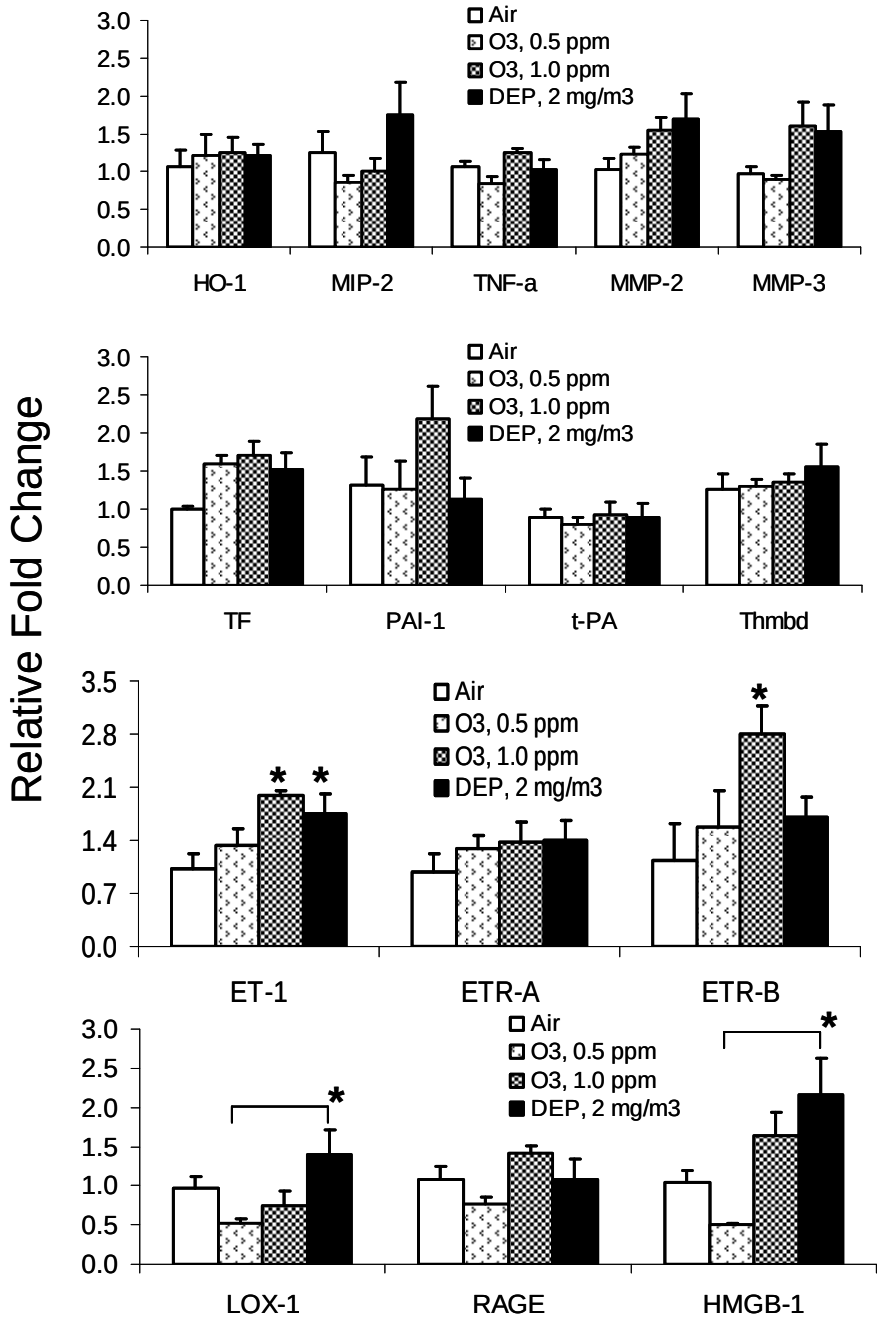
Supplemental Material, Figure 5 Legend: Pulmonary inflammation and injury following acute 2-day exposure to ozone or DEP as determined by analysis of bronchoalveolar lavage fluid (BALF). BALF was assessed for A) total cells, macrophages and neutrophils while injury was assessed by analysis of B) protein, albumin, and lactate dehydrogenase activity. Each bar represents the mean ± SEM for 6 animals. One-way ANOVA followed by Holm-Sidak method was used for total cell count, protein, and LDH activity data while Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's test was employed for albumin and neutrophil data. *Indicate significant exposure effect when compared to air controls ($P \leq 0.05$).

Supplemental Material, Figure 6



Supplemental Material, Figure 6 Legend: Pulmonary mRNA expression of biomarkers of oxidative stress, inflammation, thrombosis, as well as LOX-1 and RAGE following acute 2-day exposure to ozone or DEP. Lung tissue mRNA expression was analyzed using real-time PCR. Expression levels were normalized to 18S ribosomal RNA transcript and then to the control value from air exposed rats for computing relative fold change. HO-1, hemeoxygenase-1; MIP-2, macrophage inflammatory protein-2; LOX-1, lectin-like receptors for oxidatively modified low density lipoproteins; RAGE, receptor for advanced glycation endproducts; PAI-1, plasminogen activator inhibitor-1; t-PA, tissue plasminogen activator. Each bar represents the mean ± SEM for 6 animals for which PCR was run in duplicate for each sample. One-way ANOVA followed by Holm-Sidak post-hoc test was used for data analysis. *Indicate significant exposure effect ($P \leq 0.05$).

Supplemental Material, Figure 7



Supplemental Material, Figure 7 Legend: Aorta mRNA expression for biomarkers of oxidative stress, inflammation, thrombosis, as well as LOX-1 and RAGE following acute 2-day exposure to ozone or DEP. Aorta mRNA expression was analyzed using real-time PCR. Expression levels were normalized to 18S ribosomal RNA and then to the control value from air exposed rats for computing relative fold change. HO-1=hemeoxygenase-1; TNF-α=tumor necrosis factor-α; MIP-2=macrophage inflammatory protein-2 (a human analog of interleukin-8); MMP-2, matrix metalloprotease-2; MMP-3, matrix metalloprotease-3; TF=tissue factor; t-PA=tissue plasminogen activator; PAI-1=plasminogen activator inhibitor; Thbd=thrombomodulin; ET-1=endothelin-1; ETR-A=endothelin receptor-A; ETR-B=endothelin receptor-B; LOX-1, lectin-like receptors for oxidatively modified low density lipoproteins; RAGE, receptor for advanced glycation endproducts; HMGB-1=high mobility group box-1. Each bar represents the mean ± SEM for 6 animals for which each sample was analyzed in duplicate. One-way ANOVA followed by Holm-Sidak method was used for group comparisons.*Indicate significant exposure effect ($P \leq 0.05$) compared to air control for ET-1 and ETR-B; or compared to 0.5 ppm ozone group for LOX-1 and HMGB-1. No exposure-related changes were seen in mRNA expression of HO-1, MIP-2, TNF-α, MMP-2, MMP-3, TF, PAI-1, t-PA; or Thmbd as shown in the upper two panels.

References

Kodavanti UP, Schladweiler MC, Gilmour PS, Wallenborn JG, Mandavilli BS, Ledbetter AD, et al. 2008. The role of particulate matter-associated zinc in cardiac injury in rats. Environ Health Perspect 116(1):13-20.

Ledbetter, A. D., Killough, P., and Hudson, G. F. (1998). A low-sample-consumption dry-particulate aerosol generator for use in nose-only inhalation exposures. *Inh. Toxicol.* 10:239–251.